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Purification and characterization of anionic peroxidases from cotton (*Gossypium hirsutum* L.)

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Anionic isoperoxidases from cotton cotyledonary leaf tissue have been purified to electrophoretic homogeneity by chromatography on ion exchange, lectin affinity, and gel filtration matrices. The enzyme was stable and active at a wide range of temperatures with optimal activity at 55°C. Maximum activity was observed when the pH was adjusted from pH 5.5 to pH 7.5. Electrophoresis on sodium dodecyl sulfate (SDS)-polyacrylamide gels indicated a relative molecular mass of 56 000 Da when the sample was treated with a disulfide reducing agent and heated. The electrophoretic mobility was altered when either the disulfide reducing agent or heat treatment was omitted. Isoelectric focusing under native conditions resolved the activity into three isozymes with isoelectric points of 4.2, 4.4 and 4.6. The K_m of peroxidase for 4-aminoantipyrine and *o*-dianisidine was 350 and 36 μM, respectively. Enzyme activity was inhibited by elevated concentrations of H_2O_2 and by disulfide reducing agents. Known inhibitors of peroxidase activity such as potassium azide, sodium cyanide and sodium sulfite inhibited cotton anionic peroxidases. The absorption spectrum showed maxima at 280 and 402 nm, indicating the presence of a heme group in the active enzyme. The extinction coefficient at 402 nm was 1.12×10^5 mol^{-1} cm^{-1}.

Key words: Gossypium; cotton; peroxidase; isozymes; cotyledon

Introduction

Peroxidase (E.C. 1.11.1.7) is a ubiquitous enzyme that reduces H_2O_2 in the presence of an electron donor. Many roles have been attributed to plant peroxidases including involvement in lignin biosynthesis [1], suberization of cells [2], cross-linking of cell wall components [3–5], host-pathogen interactions [6,7], and auxin oxidation [8].

Multiple forms (isozymes) of peroxidase are known to exist in many plant tissues [9]. Both anionic and cationic forms of the enzyme are usually present. Recent work using tobacco suspension culture cells has suggested that anionic isoforms are localized in cell walls and basic peroxidases are localized in the vacuole [10]. This localization may not be universal since peanut suspension cultures release cationic peroxidases into the culture medium [11]; however, predominantly anionic forms of peroxidase are released during culture of immature cotton ovules [12,13]. How peroxidase isoforms are targeted to sub-cellular compartments is unknown. Detailed analysis of protein sequences and characterization of post-translational modfications of the various isoforms may help resolve the issue of tissue and cell localization. Recently, transgenic tobacco plants with increased levels of anionic peroxidase expression have been described [14]. Manipulation of peroxidase levels by stable introduction of the gene with a strong promoter allows the direct assessment of the role(s) of peroxidase in plant development.

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We are interested in the potential role of anionic peroxidase in two areas of cotton growth and metabolism not easily addressed by the tobacco or peanut cell culture model systems. First, cotton fibers are individual, synchronously elongating cells. As such, cotton fibers represent an ideal tissue in which to investigate the role of anionic peroxidases in cross-linking cell wall components. Second, the stimulation of anionic peroxidase production in ovule cultures challenged with *Aspergillus flavus* [13] suggests that analysis of the regulatory components of peroxidase synthesis and secretion could provide clues for the development of specific targets against these aflatoxin-producing fungi. In order to physically characterize the enzyme and develop probes to test for its function, we have purified and characterized anionic peroxidases from cotton.

Although anionic peroxidases are produced in cotton ovule culture filtrates in amounts sufficient for some physical characterization [12,13], the preparative time required for initiating ovule cultures is much greater than the time necessary to establish young germinating seedlings. The work described in this report was undertaken to determine if peroxidase could be purified to homogeneity from cotton cotyledonary leaves, and if so, to document some of the physical properties of the enzyme.

Materials and Methods

Plant material

Cotton seeds (*Gossypium hirsutum* L., Texas Marker-1) were germinated in plastic trays containing moist perlite at 30°C with constant illumination (26 μE m^{-2} s^{-1}). Cotyledonary leaf tissue was harvested at 8 days after planting.

Chemicals

QMA-Accell Plus was purchased from Millipore Waters Chromatography Division (Milford, MA). Concanavalin A-Sepharose and Sephacryl 300 were obtained from Pharmacia (Piscataway, NJ). Phenylmethylsulfonylfluoride (PMSF), 4-aminoantipyrine, 2-[*N*-morpholino]ethanesulfonic acid (MES), *o*-dianisidine, polyvinylpolypyrrolidone (insoluble) and dithiothreitol (DTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Electrophoresis chemicals and Bradford protein assay kit were purchased from BioRad (Richmond, CA). Unless otherwise noted, all other chemicals were reagent grade.

Chromatography

All columns were prepared and pre-equilibrated according to manufacturers' product literature.

Protein assay

Prior to affinity chromatography on Concanavalin A-Sepharose, protein concentrations were determined by the Coomassie Blue dye binding method of Bradford [16] using bovine gamma globulin as a standard. For later stages in the purification, a direct UV absorption method was used [17].

Peroxidase assay

Enzyme activity was determined by the rate of oxidation of 4-aminoantipyrine [18]. The standard assay contained 0.1 M potassium phosphate buffer (pH 6.5), 0.5 mM H_2O_2, 1.0 mM 4-aminoantipyrine, 0.1 M phenol, and 5–30 μl enzyme in a reaction volume of 1.0 ml. Unless otherwise noted, assays were conducted at 55°C, the optimum temperature found for cotton anionic peroxidases, and are the average of three replicates. Absorbance was monitored for 5 min at 510 nm in a Hewlett–Packard 8450A diode array spectrophotometer. Rate calculations were taken from the linear portion of the reaction, between 1 and 5 min. One nanokat of enzyme activity corresponds to the decomposition of 16.67 nmol of H_2O_2 per second. For comparison, *o*-dianisidine was also used as an electron donor in enzyme kinetic assays [19]. For assays in which *o*-dianisidine was used, the H_2O_2 concentration in the standard assay was 1.0 mM.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Electrophoresis was conducted on mini (10 × 12 cm) slab gels (Idea Scientific, Corvallis, OR) of 10% polyacrylamide according to the procedure of Laemmli [20]. Proteins were detected by silver staining the gels [21]. Molecular mass was estimated by comparing the electrophoretic mobility to standards of 95, 68, 43, 36, 29 and 18 kDa.

Isoelectric focusing

Conditions for isoelectric focusing and measuring the pH gradient were identical to those described by Mellon and Lee [22]. Samples were applied to ultrathin polyacrylamide gels by filter paper wicks. Peroxidase activity in the gels was determined by staining with 3-amino-9-ethylcarbazole.

Results and Discussion

Purification of peroxidase from cotton leaves

Enzyme extraction. Leaf tissue (40 g) was ground in a cold Waring Blendor in 300 ml of 0.2 M potassium phosphate buffer (pH 6.3) with 1 mM phenylmethylsulfonylfluoride. Unless otherwise noted, all procedures were carried out at 4°C. After the addition of solid polyvinylpolypyrrolidone (20 g), the mixture was centrifuged for 30 min at 13 000 × *g*. The supernatant liquid was filtered through Miracloth (Calbiochem, La Jolla, CA). Material precipitating with ammonium sulfate between 35 and 90% saturation was collected by centrifugation, resuspended in 25 ml of 20 mM 2-[*N*-morpholino]ethanesulfonic acid (MES)–KOH (pH 5.5), and exhaustively dialyzed against the same buffer. Insoluble material was removed by centrifugation at 12 100 × *g*.

QMA-Accell Plus chromatography. The ammonium sulfate fraction was applied to a QMA-Accell Plus column (18 × 2.8 cm) in 20 mM MES–KOH (pH 5.5), at a flow rate of 70 ml/h. The column was washed with three column volumes of starting buffer and 6-ml fractions were collected. Bound proteins were eluted by development with a 500 ml linear gradient of 0–0.1 M NaCl in starting buffer. Peroxidase activity was eluted from the column at 0.075 M NaCl.

Concanavalin A-Sepharose chromatography. Active fractions from the QMA-Accell Plus column were concentrated to 7.5 ml with a Centriprep 10 microconcentrator (Amicon Div., Danvers, MA). Buffer in the sample was exchanged by repeated concentration and dilution in concanavalin A buffer (Con A buffer) containing 50 mM Tris–HCl (pH 7.0), 100 mM NaCl, 1.0 mM $CaCl_2$, and 1.0 mM $MnCl_2$ for a total of ten cycles. The equilibrated sample was applied to a Con A-Sepharose column (12 × 0.8 cm) at a flow rate of 60 ml/h. The column was washed with four column volumes of Con A buffer, followed by a linear 50-ml gradient of 0–0.1 M α-methylmannoside in Con A buffer. No peroxidase activity was detected prior to the addition of α-methylmannoside indicating that virtually all forms of the enzyme are glycosylated. A peak of activity was eluted at 0.025 M α-methylmannoside, however, low levels of residual activity were eluted from the column throughout the remainder of the gradient. Fractions in the activity peak were concentrated with Centriprep 10 microconcentrators.

Sephacryl 300 gel filtration chromatography. After concentration, fractions from the Con A Sepharose column were applied to a Sephacryl 300 gel filtration column (50 × 2.5 cm) in Con A buffer at a flow rate of 43 ml per hour. The column was washed with four column volumes of Con A buffer. Samples containing 1.0 mg/ml protein were stored at −20°C for three months without significant loss of enzyme activity. Some samples were dialysed against 10 mM ammonium bicarbonate, lyophilized from water three times, and stored as a desiccated dry powder. A summary of the purification steps is shown in Table I. The yield using this procedure was approximately 350 μg.

Physical properties of cotton anionic peroxidases

Temperature and pH optima. Activity of cotton anionic peroxidases (Sephacryl 300 peak fraction) was measured in the standard assay from 25 to 65°C in a Hewlett–Packard 89100A spectrophotometer temperature controller. The enzyme was active in a very broad range with optimal activity at 55°C (Fig. 1). Between 60 and 65°C, activity declined to 3% of the optimum activity. To determine the effect of varying pH on cotton anionic peroxidases, the pH of the standard reaction was changed by using the following buffers: 0.2 M sodium acetate buffer was used for assays between pH 4.5 and pH 5.5; 0.1 M potassium phosphate buffer was used for assays between pH 5.5 and pH 8.0; and 0.2 M Tris–HCl buffer was used between pH 7.5 and pH 9.0. The activity was optimal over a broad range from pH 5.5 to pH 7.5.

Table I. Purification of cotton leaf peroxidase.

Step	Vol (ml)	Total protein (mg)	Total enzyme (μkat)	Specific activity (μkat/mg)	Fold purification	Yield (%)
Crude homogenate	300	318	124.5	0.39	1.0	100
$(NH_4)_2SO_4$ fraction	250	99	67.5	0.68	1.7	54.2
QMA Accell plus	80	9.4	57.5	6.12	15.6	46.2
Con A-sepharose	4	3.9	34.6	8.87	22.7	27.8
Sephacryl 300	1	0.35	14.8	42.28	108.4	11.9

As a group, cotton anionic peroxidases exhibited broad pH and temperature optima suggesting that the enzymes may remain active over a wide range of environmental conditions. It is possible that individual purified isozymes could have activity optima with a narrower range.

Electrophoresis. SDS-polyacrylamide gel electrophoresis of the Sephacryl 300-purified fractions yielded a single major band migrating at 56 kDa when the sample was treated with a reducing agent and heated (Fig. 2). In the absence of both heat treatment and DTT, cotton peroxidase migrated at 71 kDa on SDS gels. Either heating cotton peroxidase for 3 min at 100°C without the reducing agent or treating the enzyme with 50 mM DTT without heat denaturation resulted in migration of the enzyme at 42 kDa. In general, peroxidases are remarkably stable enzymes. We propose that part of this stability is due to the carbohydrate component of the glycoenzyme. Unless heated and reduced, carbohydrate moieties on the native protein might alter sodium dodecyl sulfate association, leading to this anomalous electrophoretic behavior. For many higher plants, peroxidase M_r has been estimated using partially denaturing conditions and an activity stain. The result reported here reinforces the need to completely denature the enzyme and use a detection system other than one

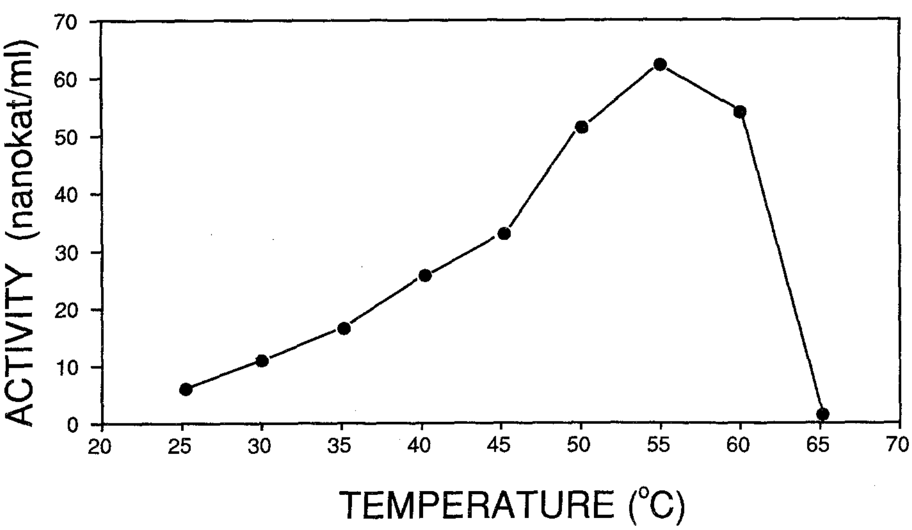

Fig. 1. Temperature optimum of cotton leaf anionic peroxidases (Sephacryl 300 fractions). Activity was measured in the standard assay as described in Materials and Methods.

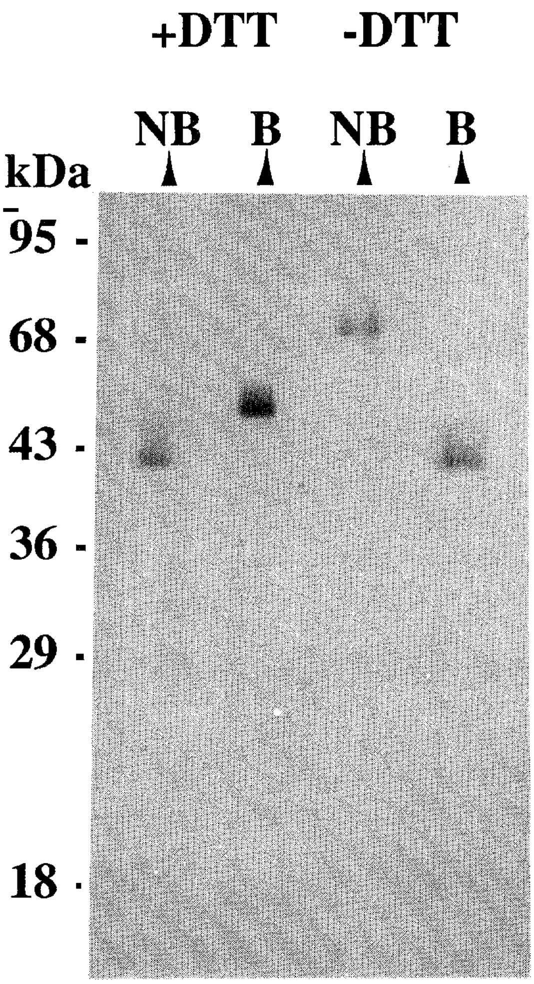

Fig. 2. SDS-PAGE of purified cotton leaf peroxidases. Each lane contains 1.5 μg of protein (Sephacryl 300 fractions). The relative molecular mass of several standard marker proteins are indicated on the left side of the gel. +DTT samples contained 50 mM DTT. −DTT samples contained no disulfide reducing agent. Samples in lanes marked with B were heated to 100°C for 3 minutes. Samples in lanes marked with NB were not heated. Bands were detected by silver staining.

based on activity to accurately estimate peroxidase molecular mass. Similar electrophoretic anomalies were reported recently for polyphenoloxidase purified from broad beans [23].

Differences in the detection of cotton peroxidase by silver staining on SDS gels were also noted. Samples subjected to electrophoresis under the most native conditions (no heat or DTT) reproducibly stained more weakly with silver than the most highly denatured samples (heated samples containing DTT). Staining of samples treated with either heat or disulfide reducing agent was intermediate. Silver staining occurs in regions rich in basic and sulfur-containing amino acids [24]. Fully denatured cotton peroxidase probably has more of these silver-reactive domains exposed

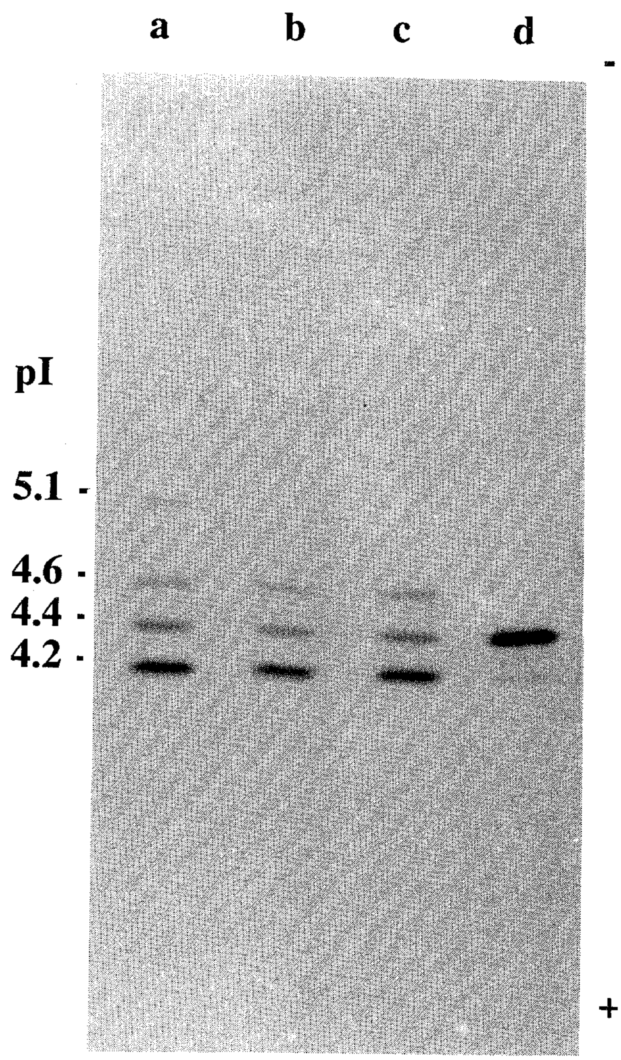

Fig. 3. A comparison of cotton leaf peroxidases at several stages of purification with cotton ovule peroxidase by isoelectric focusing under non-denaturing conditions. Crude homogenate (a), QMA-Accell fractions (b), Con A-Sepharose fractions (c), cotton ovule peroxidase (d) [17]. Peroxidase activity was visualized with 3-amino-9-ethylcarbazole.

than non-denatured or partially-denatured protein.

Isoelectric focusing. Three anionic peroxidase isozymes were evident by isoelectric focusing of fractions at various stages of purification (Fig. 3). The most abundant isozyme had an isoelectric point of 4.2. A second isozyme (p*I* 4.4) co-migrated with peroxidase purified from cotton ovule culture media [16]. A third isozyme migrated with a p*I* of 4.6. A fourth isozyme (p*I* 5.1) was evident in crude cell homogenates, but was not detected on activity-stained isoelectric focusing gels after the $(NH_4)_2SO_4$ precipitation step. The number of reported peroxidase isozymes in different plant tissues varies greatly [24]. In some cases, failure to take appropriate precautions against phenolic oxidation leads to artifactual generation of isozymes [25]. It is encouraging to note that only four anionic isozymes were detected by isoelectric focusing of the cotton leaf crude cellular homogenate. Factors leading to either the activity loss or the physical loss of the p*I* 5.1 form after $(NH_4)_2SO_4$ precipitation remain unknown. At this point, it is unclear whether the isozymes in cotton leaf represent distinct gene products or post-translational modifications of a single gene product. Efforts to separate the isozymes with chromatofocusing and characterization of the carbohydrate components will help resolve this issue.

Enzyme kinetics. Two artificial electron donors, 4-aminoantipyrine and *o*-dianisidine were tested in experiments to assess the affinity of cotton anionic peroxidase (Sephacryl 300 fractions) for substrate. The K_m for aminoantipyrine was estimated to be 350 μM and the K_m for *o*-dianisidine was 36 μM. Although 4-aminoantipyrine was not as effective as *o*-dianisidine as a substrate, safety and waste disposal considerations recommended its use in routine assays. Peroxidase activity was optimal at 0.5 mM H_2O_2 when aminoantipyrine was used (Fig. 4A) and at 1.0 mM when *o*-dianisidine was used as the electron donor (Fig. 4B). High concen-

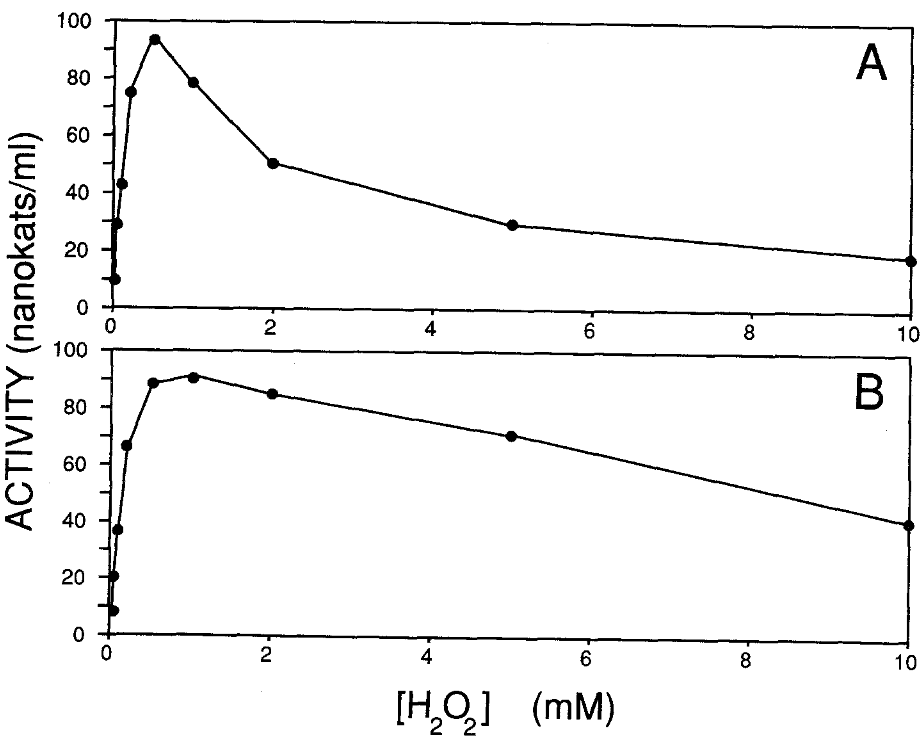

Fig. 4. Optimum H_2O_2 concentration for cotton anionic peroxidases. Activity of anionic peroxidases (Sephacryl 300 fractions) was determined in the presence of varying concentrations of H_2O_2 with (A) 4-aminoantipyrine or (B) *o*-dianisidine as the electron donor.

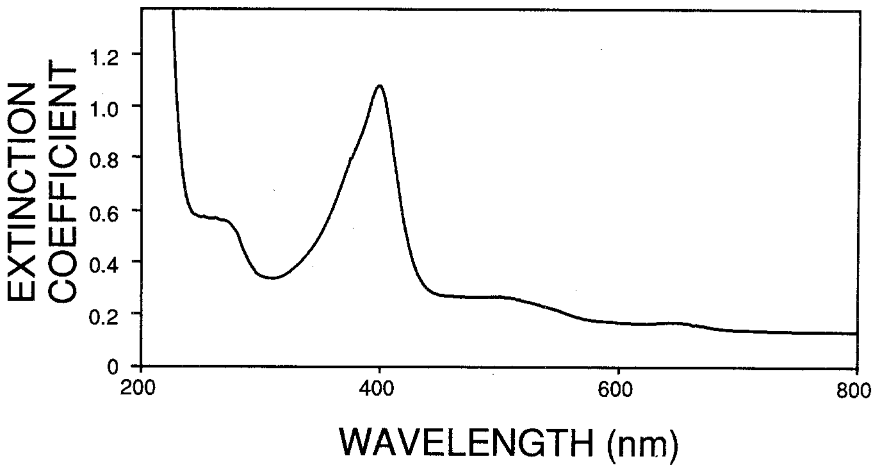

Fig. 5. The extinction coefficient (× 10^5 mol^{-1} cm^{-1}) of cotton leaf anionic peroxidase (Sephacryl 300 fractions) at 220–800 nm. The sample contained enzyme in 0.1 M potassium phosphate (pH 6.5). Protein concentration was 150 μg/ml.

trations of H_2O_2 inhibited cotton anionic peroxidase activity.

Sensitivity to inhibitors. Several compounds were inhibitory to peroxidase activity when added to the standard enzyme assay mixture prior to enzyme addition. Both KCN and Na_2SO_3 were completely inhibitory to the enzyme at 10 μM, whereas NaN_3 was not completely inhibitory until 100 μM. Enzyme activity was also sensitive to the presence of disulfide reducing agents. The enzyme was completely inhibited in 200 μM dithiothreitol, β-mercaptoethanol, or dithioerythritol.

Absorption spectrum. Purified peroxidase in 0.1 M potassium phosphate (pH 6.5), was used for determining the absorption maxima between 220 and 800 nm (Figure 5). Two maxima were evident, one at 402 nm and another at 280 nm. The extinction coefficient at 402 nm was 1.12 × 10^5 mol^{-1} cm^{-1}.

Young cotyledonary leaf tissue from cotton is a rich source of anionic peroxidase isozymes. In order to assess the role of peroxidase in cotton fiber growth and in *A. flavus* infections, a rapid and sensitive method for monitoring peroxidase synthesis and transport is required. In preliminary studies, commercial antibody preparations directed against horseradish peroxidase isozyme C were poor reagents, in Western blot assays, against cotton peroxidase. Initial attempts at producing antibodies to cotton anionic peroxidases from ovule cultures resulted in a preparation that recognized predominantly (>98%) the carbohydrate portion of the glycoprotein. We expect to use chemically-deglycosylated anionic peroxidase from cotton leaf tissue as an immunogen to elicit polyclonal antibodies in rabbits. Continued physical characterization of cotton anionic peroxidase isozymes, including amino acid and carbohydrate composition, peptide mapping and sequencing will enable us to design probes for molecular characterization of this enzyme in cotton.

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